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Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The HER-2/neu extracellular domain (ECD) is shed from breast carcinoma cells in culture and is found at elevated levels in sera of patients with metastatic breast cancer where it may predict recurrence. Our studies show that an N-terminally truncated HER-2/neu product, p95, is produced when the ECD is shed, that p95 has kinase activity, and is expressed to a greater extent in breast cancer patients with lymph node metastasis. **Purpose**. The objective of this proposal is to directly test the hypothesis that shedding of the extracellular domain of HER-2/neu, which creates the truncated p95 kinase, promotes oncogenesis. **Scope**. The effect of shedding to oncogenesis will be examined by further characterizing the control of shedding and genetically altering shedding activity to test the impact on tumorigenesis and oncogenesis. **Results**: We have developed and characterized several strategies for generation of mutants of p185HER-2 to alter shedding and have investigated approaches to modulate shedding by treatment with exogenous effectors. We have been unsuccessful in development of mutants that specifically alter shedding, but that do not affect other receptor activities. These mutant proteins either are unstable or have altered kinase activity. We conclude that juxtamembrane mutants are problematic for examining the function of shedding on receptor-mediated tumorigenesis. Efforts to use approaches that employ chemical inhibitors and activators of shedding to examine the impact on transformation and tumorigenesis have not yielded interpretable results that distinguish the effects on receptor activity versus receptor shedding. Moreover, these modulators, alone, appeared to affect cell behavior. We conclude that modulation of shedding cannot be clearly separated from direct effects on the activity of the receptor itself or from secondary effects on the cells. We conclude that future studies need to be conducted on the p95 in human breast cancer samples to determine whether it predicts outcome in patients. While the epidemiological study may not yield proof that p95 causes metastasis and poor outcome, ultimately this line of study may be more beneficial to breast cancer patients than in vitro analyses that are subject to numerous problems with interpretation as determined in this project.

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INTRODUCTION

Subject: The HER-2/neu extracellular domain (ECD) is shed from breast carcinoma cells in culture and is found at elevated levels in sera of patients with metastatic breast cancer where it may predict poor prognosis, response to adjuvant endocrine and chemotherapy, and allow tumor cells to escape immune surveillance. Our studies show that an N-terminally truncated HER-2/neu product, p95, is produced when the ECD is shed, has kinase activity, and is expressed to a greater extent in breast cancer patients with lymph node metastasis (1). **Purpose:** The objective of this proposal is to directly test the hypothesis that shedding of the extracellular domain of HER-2/neu and creating of the truncated p95 kinase promotes oncogenesis. **Scope:** To examine the impact of shedding on oncogenesis: (a) We will genetically alter shedding of HER-2/neu. To alter shedding, deletion and domain replacement mutants will be constructed within the HER-2/neu juxtamembrane cleavage domain. The mutations to be made will be based on known structural determinants of shedding defined through studies of diverse transmembrane proteins. A second approach to genetically alter shedding will be pursued by expressing HER-2/neu in cells that are null for shedding enzyme. (b) The impact of altered shedding to oncogenesis will be examined in cell culture and animal models. The transforming activity of HER-2/neu with genetically altered levels of shedding will be examined by well-established cell culture models of transformation, by tumorigenesis assays in nude mice, and by metastatic potential in immune compromised mice.

BODY

STATEMENT OF WORK

The following outlines the statement of work to be conducted and the progress we have made in this direction.

Task 1. Genetically alter the proteolytic shedding of p185HER-2/neu (months 1-24).

To examine the impact of shedding of the ectodomain of p185HER-2 on tumorigenesis, we proposed to alter the extent of shedding by engineering altered sequence in the juxtamembrane domain of p185HER-2. The juxtamembrane region of the ectodomain is known to be the site of proteolytic cleavage of transmembrane proteins during shedding (Arribas et al., 1996). The mutants were then to be characterized first for their proper location at the cell surface and for kinase activity by examining autophosphorylation of the mutant receptor and by analyzing the kinase enzymatic activity of the receptor by immunoprecipitation and in vitro kinase activity. The characterization of the mutant receptor was an important first step since alterations in biosynthesis, processing, or kinase activity would be expected to affect tumorigenic potency of the receptor obscuring effects of shedding.

In the first and second years, a deletion of the juxtamembrane stalk of p185HER-2 was constructed and secondly an altered sequence of the juxtamembrane stalk was created by swapping with the juxtamembrane of tumor necrosis factor-α precursor (preTNFα), which undergoes potent tumor promoter inducible shedding. From this study it was concluded that deletion of the juxtamembrane of p185HER-2 protein resulted in folding defects leading to deficient protein production. We next designed a strategy to delete the 16 amino acids adjacent to

the transmembrane domain, to exactly mimic an alternative splicing event described by Siegel et al., (1999), which results in deletion of an exon. Further biochemical characterization of this variant product of HER-2 has revealed that the protein has enhanced in vitro kinase activity. Altered kinase activity and enhanced transforming activity of this mutant protein precludes efforts to examine effects of altered shedding on tumorigenesis, since any effect of shedding would be secondary to altered kinase activity. Another plan for generating a mutant with altered shedding activity was to swap the juxtamembrane domain of tumor necrosis factor-α precursor (preTNFα) with that of p185HER-2. The shedding of TNFα is efficiently induced by addition of tumor promoters (Blobel, 1997). In contrast p185HER-2 shedding is very slow and is not inducible by tumor promoters (Christianson et al., 1998, Codony-Servat et al., 1999). To examine the properties of this mutant, we performed transient transfection into Cos-7 cells. The protein was produced and detected by immunoblotting. To further characterize this mutant, we examine the kinase activity by examining the tyrosine phosphorylation level by conducting anti-phosphotyrosine blots. In this system, overexpression of the wildtype p185HER-2 results in dimerization and constitutive tyrosine phosphorylation. Comparisons of the tyrosine phosphorylation level of the mutant and wildtype have initially revealed that the mutant has decreased levels suggested that the kinase activity is altered. Further characterizations of this mutant conducted in the last year of funding further determined that effects on altered shedding could not be distinguished from direct effects of the mutation on the receptor activity. Moreover, the effects on shedding on cell proliferation and transformation were further complicated by the cellular effects of the tumor promoters that are required to induce shedding.

In summary, the juxtamembrane region of p185HER-2, which is the site of proteolytic cleavage to achieve shedding is also a region of the receptor that affects receptor activity. Consequently, it is difficult, if not impossible to distinguish effects on shedding versus effects on receptor activity. The problems involved in interpretation of results obtained using juxtamembrane mutations are further emphasized by recent studies (Burke and Stern, 1998) showing the importance of the juxtamembrane domain sequence of p185HER-2 in dimerization and receptor activation. This study suggests that any attempts to disrupt the juxtamembrane region will have the primary consequence of affecting receptor activation.

In the final year of funding we tested alternative methods to alter shedding. though the use of cleavage inhibitors and activators that used alone and in combination. For inhibitors, we tested TAPI (Christianson et al., 1998) and BB-94 (Codony-Servat et al., 1999) both metalloprotease inhibitors shown to inhibit HER-2 shedding. To stimulate shedding, we used the 4-aminophenylmercuric acetate (APMA), a matrix metalloprotease activator known to cause potent stimulation of HER-2 shedding (Molina et al., 2001). HER-2 transfected 3T3 cells and control 3T3 cells were treated with 10 μM TAPI, and as previously observed, shedding of the ectodomain and production of p95 were inhibited. We then tested the effects of TAPI on the growth properties of the cells. For the focus forming assay, the 3T3/HER-2 cells were plated at low density in 12 well plates. For the anchorage independent growth assays, the cells were plated in soft agar. Triplicate wells were treated with 10 μM TAPI or with the control vehicle. As a futher control, the parental 3T3 cells were also treated in parallel experiments with 10 μM TAPI. While TAPI appeared to have a slight (~30%) inhibitory effect in both assays, the parental 3T3 cells were similarly affected in the focus forming assay. Therefore, the effects of TAPI appeared to be nonspecific for HER-2 shedding. Next we investigated the effects of the metalloprotease activator APMA on shedding, focus formation, and anchorage independent growth of parental and 3T3/HER-2 cells. As previously reported by Molina et al., 2001), APMA strongly stimulated shedding. However, as observed when TAPI was employed, the effects of APMA on the parental and HER-2 cells were comparable. There appeared to be no specificity

for the 3T3/HER-2 cells. For the final year of study, we also proposed to conduct a similar set of studies employing MCF-7 breast carcinoma cells. The MCF7 cells were stably transfected with HER-2 and several colonies were characterized for HER-2 expression. After analysis of ~ 15 clones of stably transfected cells, the maximum level of HER-2 overexpression achieved was 3 fold. Despite this small increase, we observed a striking 15 fold increase in Heregulin mediated tyrosine phosphorylation of HER-2. We next measured the shedding of HER-2 by the amount of ECD released in the culture media determined by ELISA, and by the level of p95 in the cell extract. Initial analyses revealed little or no shedding in these cells. Next, we tried to enhance shedding by treatment with the APMA metalloprotease activator. Still, there was no detectable shedding from these cells. A recent publication further indicated that the MCF7 cells are defective in shedding activity. Consequently, we were unable to determine the effects of altered shedding on the behavior of these cells.

KEY RESEARCH ACCOMPLISHMENTS

In the final year of funding we:

- Further analyzed juxtamembrane domain swapping mutants of HER-2 for kinase activity and for intracellular signaling
- Characterized the effects of TAPI and APMA metalloprotease activator on shedding from 3T3/HER-2 cells
- Tested the effects of TAPI and APMA on the focus forming activity of parental versus 3T3/HER-2 cells
- Tested the effects of TAPI and APMA on the anchorage independent growth of 3T3/HER-2 cells
- Generated stable HER-2 overexpressing MCF7 breast carcinoma cell lines
- Characterized the MCF7/HER-2 cells for HER-2 levels and for HER-2 mediated signal transduction
- Tested the MCF7/HER-2 cells lines for shedding by measuring the ECD and p95
- Treated MCF7/HER-2 cells with APMA and tested shedding and p95 production.

REPORTABLE OUTCOMES

- Manuscripts, abstracts, presentations:
  None.
- Patents and licenses applied for and/or issued:
  None
- Degrees obtained that are supported by this award:
  None
- Development of cell lines, tissue, or serum repositories:
  None
- Informatics such as databases and animal models:
  None
- Funding applied for based on work supported by this award:
  None
- Employment or research opportunities applied for and/or received :
  None

CONCLUSIONS

To summarize, we have employed several different strategies for experimental modulation of shedding in order to test effects on tumorigenic activity of HER-2. First we used a genetic approach and constructed deletions mutants and domain substitution mutants to alter the extent of shedding. Because of altered receptor processing and kinase activity caused by these mutations and recent information suggesting that the juxtamembrane region of p185HER-2 is critical for receptor dimerization and kinase activity, we concluded that juxtamembrane mutants will not be an effective approach for testing the role of shedding of the ectodomain in receptor mediated tumorigenesis. Next, we used additional approaches involving the use of chemical inhibitors and stimulators of shedding for testing the effects of shedding. While we determined that metalloprotease inhibitors and activators were effective in modulating the extent of shedding, these effectors also had an effect on cell growth that was independent of HER-2 shedding. Therefore, the effect of these effectors on shedding versus other secondary effects on the treated cells could not be distinguished.

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ADDENDUM: Addendum

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Research conducted in the prior three years of funding led us to conclude that modulation of shedding by genetic alteration of the receptor cannot be clearly separated from direct effects on the activity of the receptor itself. We next considered that the *in vitro* assays used to test the effects of shedding including anchorage-dependent and independent growth are unsuitable for assessing impact on tumor progression and metastasis. This point is further emphasized by recent findings in our laboratory (Molina et al., *Clinical Cancer Research,* 8:347-353, 2002) demonstrating that p95HER-2 in primary breast cancers correlates with lymph node metastasis and that the level of p95HER-2 is enhanced in metastatic nodes compared to primary tumor tissue. These results indicate that p95HER-2, created by shedding, may be involved in metastasis, whereas the full-length receptor, p185HER-2 may be more important in the initial stages of tumor formation and proliferation. In order to further evaluate the impact of p95HER-2 on breast cancer metastasis, it is important to have a model in which HER-2 overexpressing mammary tumors undergo metastasis. While the nude mouse model using human breast cancer xenografts can be used to assess tumorigenesis, metastasis does not occur in this model. However the c-neu MMTV transgenic mice develop mammary tumors that metastasize to the lung (Guy, C.T., M.A. Webster, M. Schaller, T.J. Parsons, R.D. Cardiff, and W.J. Muller, *Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease.* Proc. Natl. Acad. Sci. U. S. A., 1992. **89**(22): p. 10578-82). This is one of the only available models to evaluate impact of HER-2 shedding on mammary tumor metastasis. However the previous MMTV-c-neu models use the neu gene, which is the rat ortholog of HER-2. Since shedding has not been demonstrate for the rat neu receptor, and since there are several differences between the rat and human receptors, we considered that creation of MMTV-HER-2 mouse would be of critical importance in future studies to determine the significance of HER-2 shedding to breast cancer.

For the 4th year funding extension, we took the initial steps required to generate a MMTV-HER-2 transgenic mouse to be used in future studies to determine the functional significance of shedding to tumor metastasis. The first step required cloning the HER-2 gene into the MMTV LTR (pMSG) plasmid purchased from Amersham/Pharmacia. This plasmid is extremely large (7.6kb), is the only available MMTV driven plasmid and has only four unique cloning sites. The HER-2 cDNA is also fairly large at 3.8 kb. Therefore, finding a cloning site in the plasmid that was not in the HER-2 cDNA was difficult. We found that an Xho site and Sal I site were unique and decided to clone the HER-2 into this site. However after several attempts, no clones were obtained probably because the restriction sites were too close to each other in the vector creating problems with complete digestion. The next strategy was to clone into the Xho site. Several weeks of cloning efforts were required possibly due to large size of the plasmid leading to self-ligation. When clones that contained HER-2 were obtained, we sequenced the HER-2 insert and found that mutations were created during the cloning procedure. We next tried mutagenesis to correct the mutations. While the mutation was corrected, three other mutations were introduced during the mutagenesis procedure. The next strategy was to cut and paste three different fragments from the original gene into the MMTV expression plasmid. This approach was successful. Finally we digested the entire HER-2 insert from the vector and recloned into original pMSG vector to eliminate mutations created in

the vector during the cloning. Sequencing of the final construct revealed an MMTV-HER-2 plasmid without mutations. Next we sought to confirm expression, inducible by dexamethasone, in transiently transfected mammalian cells. The expression plasmid was transiently transfected into HEK cells that were serum starved for 16hrs and then treated with control vehicle or different concentrations of dexamethasone. In 24 hours the transfected cells were extracted and analyzed by Western blot analysis for dexamethasone induced expression of HER-2. The results, shown in Fig. 1 below, demonstrated induction of HER-2 from this expression plasmid. The next step will be in collaboration with Dr. Manfred Braetscher at OHSU, Director of the Transgenic Facility. Dr. Braetscher will inject the MMTV-HER-2 expression plasmid into embryos. Future experiments, outside the scope of this no cost extension, will involve generation of the transgenic animals, analysis of mammary tumor formation, and investigation of shedding of the extracellular domain and presence of p95HER-2 in primary and metastatic tumors. This model will be useful in the future to test the effects of shedding inhibitors.

Figure 1. Dexamethasone induced expression of p185HER-2 from the MMTV-HER-2 expression plasmid.